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Total number of authors:
23

Published in:
American Journal of Physiology: Endocrinology and Metabolism

Link to article, DOI:
[10.1152/ajpendo.00363.2015](https://doi.org/10.1152/ajpendo.00363.2015)

Publication date:
2016

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Kiillerich, P., Myrmel, L. S., Fjære, E., Hao, Q., Hugenholtz, F., Sonne, S. B., Derrien, M., Pedersen, L. M., Petersen, R. K., Mortensen, A., Licht, T. R., Rømer, M. U., Vogel, U. B., Waagbø, L. J., Giallourou, N., Feng, Q., Xiao, L., Liu, C., Liaset, B., ... Kristiansen, K. (2016). Effect of a long-term high-protein diet on survival, obesity development, and gut microbiota in mice. *American Journal of Physiology: Endocrinology and Metabolism*, 310(11), [E886-99]. <https://doi.org/10.1152/ajpendo.00363.2015>

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**Effect of a long-term high-protein diet on survival,
obesity development and gut microbiota in mice**

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Author Contributions:

UBV, LM and KK designed the experiment. PK, LSM, EF, QH, FH, SBS, MD, LMP, RKP, AM, TRL, MUR, MK, LJW, NG, QF, LX, CL, WJ and BL performed the experiments and analyzed data. PK, LSM, FH, LM and KK wrote the manuscript; all authors read, commented and approved the final version of the manuscript.

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30 **Running head:** Long-term high-protein diet and obesity

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36 **Abstract**

37 Female C57BL/6J mice were fed a regular low fat diet or high fat diets combined with either
38 high or low protein:sucrose ratios during their entire lifespan to examine the long-term effects
39 on obesity development, gut microbiota and survival. Intake of a high fat diet with a low
40 protein:sucrose ratio precipitated obesity and reduced survival relative to mice fed a low fat
41 diet. By contrast, intake of a high fat diet with a high protein:sucrose ratio attenuated life-long
42 weight gain, adipose tissue expansion, and survival was not significantly altered relative to
43 low fat fed mice. Our findings support the notion that reduced survival in response to high fat
44 high sucrose feeding is linked to obesity development. Digital gene expression analyses,
45 further validated by qPCR, demonstrated that the protein:sucrose ratio modulated global gene-
46 expression over time in liver and adipose tissue modulating pathways related to metabolism
47 and inflammation. Analysis of fecal bacterial DNA using the Mouse Intestinal Tract Chip
48 revealed significant changes in the composition of the gut microbiota in relation to host age
49 and dietary fat content, but not the protein:sucrose ratio. Accordingly, dietary fat rather than
50 the protein:sucrose ratio or adiposity is a major driver shaping the gut microbiota, whereas the
51 effect of a high fat diet on survival is dependent on the protein:sucrose ratio.

52

53 **Keywords:** High protein diet, high fat diet, survival, obesity, gut microbiota

54 **Introduction**

55 Intake of high fat diets have been associated with the development of obesity and several
56 metabolic dysfunctions, including insulin resistance, hepatic steatosis, hyperlipidemia and low
57 grade systemic inflammation (42). Diets with more than 30% energy originating from fat
58 promote obesity in mice, and high fat diet-induced obesity is a frequently used rodent model
59 for studies on obesity and related metabolic disorders (15). However, these models can not be
60 used to distinguish whether the observed metabolic dysfunctions result from the obese state or
61 from the high fat feeding. Rodent studies have shown that obesity is prevented if the increase
62 in dietary fat is accompanied by a high protein:sucrose ratio (12, 14, 30, 32, 33, 36). This may
63 reflect higher satiety and diet-induced thermogenesis when the protein content in the diet is
64 increased (31). Conversely, reducing the fat content from 40 to 30 energy% is sufficient to
65 counteract insulin resistance in rodents (16). Together with the finding that glucose
66 intolerance and insulin resistance are detectable within the first week of high fat feeding, these
67 findings suggest that development of insulin resistance and glucose intolerance may be
68 directly related to the dietary fat content and not obesity (50).

69 Even though a high intake of fat may lead to metabolic disturbances prior to the onset of
70 obesity, it is generally acknowledged that obesity reduces longevity (2) and is associated with
71 increased all-cause mortality in humans (11). It is also well documented that energy
72 restriction increases, whereas high fat feeding reduces lifespan in rodents, but in most
73 experiments it has been difficult to separate the beneficial effects of caloric restriction *per se*
74 from that of leanness (2). However, the finding that longevity is increased in feed restricted
75 *ob/ob* mice, despite high levels of obesity suggests that longevity is related to feed intake
76 rather than the state of obesity (17). On the other hand, the increased longevity in fat-specific
77 insulin receptor knockout mice that are protected against diet- and age-induced obesity,
78 despite normal feed-intake, suggests that the reduced fat mass, and thereby, the possibly

79 reduced burden of obesity related disorders may be of importance (2). Moreover, increasing
80 the protein:sucrose ratio increased the longevity in high fat diet fed mice, and conversely,
81 decreased longevity was observed in response to a diet with a low protein:sucrose ratio, which
82 was also linked to a high rate of weight gain early in life (22). Still, median lifespan in high
83 fat-high protein fed mice was reduced compared with low fat fed mice, and these findings
84 underscore the importance of macronutrient composition. Interestingly, a recent article using a
85 complex geometric framework for nutrition concluded that the ratio of macronutrients, and
86 not caloric intake was determining various metabolic parameters as well as longevity (43).

87 Reduced biodiversity and compromised stability of the intestinal microbiota have been
88 reported in elderly humans (46), and modulation of the gut microbiota has been suggested as a
89 modality for longevity extension (34). Dietary composition (8, 19, 47, 49, 51) and the state of
90 obesity (26) are also correlated to the microbial composition in the gut, and recent studies
91 indicate that obesity correlates with decreased microbial gene richness (24). Accumulating
92 evidence indicates that the gut microbiota contributes to the development of diet-induced
93 obesity (1, 8, 40, 47, 48), and additionally, influences the development of metabolic
94 dysfunctions associated with obesity (5, 24, 37).

95 The linkage between diet, obesity and gut microbiota is, however, not elucidated. On one
96 hand, the findings that conventionalization of germ-free mice with microbiota from both diet-
97 induced (47) and genetically obese mice fed regular chow (1) resulted in increased weight
98 gain compared to mice transplanted with a gut microbiota from lean controls suggests that
99 obesity drives transferable changes in the gut microbiota inducing weight gain and adiposity.
100 By contrast, high fat feeding studies using obesity resistant RELM β KO mice suggested that
101 dietary factors are more relevant for microbiome composition than obesity (19).

In this study we investigated the long-term effect of high fat diets with low and high protein:sucrose ratios on survival, gut microbiota, and the development of obesity and related metabolic disorders in mice. We also hypothesized that this approach would enable us to distinguish whether the observed effects on the gut microbiota reflected the obese state or the high fat feeding.

Materials and Methods

Mice and feeding. 150 female C57BL/6JBomTac mice (3 weeks of age, Taconic Europe, Ejby, Denmark) were divided into 3 experimental groups with 50 mice in each, with an overall equal body weight mean for all the groups. Within each group mice were randomly divided into 10 cages with 5 animals in each. Mice were kept at 55±5% humidity, 22±1°C, in a 12:12 light:dark cycle.

Mice were fed *ad libitum* with a low fat reference (REF) diet or a corn-oil based high-fat diet (25%) enriched with either protein or sucrose (43%), all obtained from Ssniff Spezialdiäten GmbH (Soest, Germany) (Table 1) throughout life. Animals were weighed, and feed and water intake were recorded once a week throughout the experiment. All mice that died or the mice which were killed because they became moribund or showed markedly disturbed general condition during the study were necropsied. At termination all the remaining mice were sacrificed and necropsied. For each feeding group the experiment was terminated when 50% of the animals had died.

Ten mice from each group were sacrificed 6 and 18 months after the start of the experiment by cardiac puncture after anesthesia. Prior to sacrifice, animals were weighed. Blood was collected and separated into plasma and RBC before freezing at -80°C. Liver, interscapular

125 brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT) and gonadal white
126 adipose tissue (gWAT) were weighed and stored at -80°C until further analyses.

127 Feces was collected from the cages during 2 days at 3, 6 and 16 months after the start of the
128 experiment.

129 The experiment was approved by the Animal Experiment Inspectorate in Denmark and was
130 conducted in compliance with the European convention for the protection of vertebrate
131 animals used for experiments and other scientific purposes (Council of Europe, no. 123,
132 Strasbourg, France, 1985).

133 **Oral glucose tolerance test.** Glucose (1.5 mg/kg body weight) was administered orally in
134 overnight fasted mice. Blood was collected from the tail vein of conscious animals at 15, 30,
135 60 and 120 minutes after glucose administration and blood glucose was measured using a
136 glucometer (Ascensia Contour, Bayer, Norway) at baseline and during the test at the indicated
137 time points.

138 **Analyses**

139 **RNA extraction and RT-qPCR.** RNA was extracted from liver, iWAT and gWAT using
140 TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized in duplicates using
141 TaqMan Reverse transcription reagents (Applied Biosystems, Foster City, CA, USA)
142 containing Multiscribe Reverse Transcriptase. Gene expression was determined in 384-well
143 reaction plates by RT-qPCR using the LightCycler 480 Real-Time PCR System (Roche
144 Applied Sciences, Basel, Switzerland). Expression of target genes was normalized to TATA
145 box-binding protein (*Tbp*) mRNA. Primer sequences are available upon request.

Tissue lipid extraction and lipid class analysis. Total lipid was extracted from liver samples with chloroform:methanol, 2:1 (v/v) and quantified on a Camaq HPTLC system and separated on HPTCL silica gel as previously described (30).

Plasma lipids. Plasma triacylglycerol and cholesterol were determined using conventional enzymatic kits (DIALAB, Austria) and a MaxMat PL II (MAXMAT S.A., Montpellier, France).

Digital gene expression profiling (DGEP) and data analysis. Tag library preparation from liver, iWAT and gWAT RNA was performed using Illumina NlaIII Gene expression sample preparation kit and sequenced using Illumina Genome Analyzer II system (BGI-Shenzhen, P.R. China) according to the manufacturer's recommendations. Image analysis, base calling and extraction of tags were performed using the Illumina pipeline. The tag entities of DGEP libraries were mapped to mRNA reference using bwa (27). The matched tags were filtered by the match position to NlaIII recognition CATG sites by custom perl scripts. Bioconductor package edgeR (41) was used to perform statistical analysis for finding genes that differed significantly in expression between diets and time points in different tissues (diets: $p \leq 0.05$, time: $FDR \leq 0.05$). GO annotation for Biological processes was performed using bioconductor package GOstats (10).

MITChip analysis. Bacterial DNA was extracted from fecal samples from 3-7 cages per diet group as previously described (18). The microbial community in fecal samples were analysed using the Mouse Intestinal Tract Chip (MITChip). This phylogenetic microarray was designed using criteria of the Human Intestinal Tract Chip (HITChip) developed by Rajilic-Stojanovic et al. (38). The MITChip consists of 3,580 different oligonucleotides specific for the mouse intestinal microbiota (13). The array targets the V1 and V6 regions of 16S rRNA genes of bacteria. The 16S rRNA genes were amplified from twenty nanogram of intestinal

metagenomic DNA with the primers T7prom-Bact-27-F and Uni-1492-R (Table 2). These PCR products were transcribed, labelled with Cy3 and Cy5 dyes and fragmented as described elsewhere (38). Finally the samples were hybridized on the arrays at 62.5°C for 16 hours in a rotation oven (Agilent Technologies, Amstelveen, The Netherlands). After washing and scanning of the slides, data was extracted with the Agilent Feature Extraction software, version 9.1. The data were normalized and analyzed using a set of R-based scripts in combination with a custom-designed relational database, which operates under the MySQL database management system.

The RPA signal intensities were taken to analyze microbiota profiles at different levels of taxonomic resolution as indicated in the text (23). Phylum level data was used to calculate the *Bacteroidetes* and *Firmicutes* levels, and differences were tested with the Student's t-test. To determine correlation of genus-like level microbial groups detected on the MITChip with a specific diet, redundancy analysis (RDA) as implemented in Canoco for Windows5 was used (4). RDA is a linear method of canonical ordination, which linearly combines explanatory variables on the ordination axis. The Monte Carlo Permutation test was used to assess the significance of the variation in the RDA's. Probe level data was used to calculate the Shannon diversity with an in-house R-script.

Statistics. All data are presented as means \pm standard error of the mean. All statistical analyses were carried out in GraphPad Prism, except Digital Gene Expression Profiling and MITChip analysis (see their respective paragraphs for details). Comparison between groups were performed on all data by one-way ANOVA followed by a Bonferroni adjusted Fisher's Least Significant Difference (LSD) test, taking into account the total number of pairwise comparisons. For repeated measures of the body weight development curve, a two-way repeated measures ANOVA was applied to detect overall effects of diet on body weight and time, and post-hoc Bonferroni adjusted pair-wise comparisons were used to detect weekly

differences between diets. Kaplan-Meier survival curves were analyzed using a log-rank test to determine significant difference in survival. In all cases a significance level of $\alpha = 0.05$ was used.

Results

A high dietary protein:sucrose ratio attenuates high fat diet-induced weight gain and mortality

The mice were fed the experimental diets (Table 1) *ad libitum* from the age of three weeks and onwards until 50% of the mice in each experimental group had died; at this point all the remaining mice were terminated. A high protein:sucrose ratio strongly attenuated high-fat diet-induced weight gain (Fig. 1A), but high fat-high protein (HFP) fed mice still gained more weight than mice fed the low fat reference (REF) diet. Mice receiving the high fat diet supplemented with sucrose (HFS) were significantly heavier than the reference group already at week 10, whereas the difference between the REF and HFP fed mice first became significant from week 53. The body weight development revealed distinct differences in growth rates during the feeding trial and growth rates for HFS fed mice were higher than HFP fed mice during week 0-8 and week 10-65. As expected, growth rates declined significantly with time for all diets ($p < 0.0001$).

Weekly and cumulative energy intakes were significantly higher in both HF groups compared to the REF group ($p < 0.0001$ for all comparisons, data not shown). The HF fed mice had a comparable energy intake during the feeding trial, with the exception of week 4-10 and week 70-76 where the HFS fed mice ate more. This resulted in a significantly higher weekly and cumulative energy intake in the HFS group compared to the HFP group ($p < 0.0039$ and $p < 0.0001$, respectively). Feed efficiencies were comparable in HFS and HFP fed mice during

the first 8 weeks of feeding (Fig. 1B). However, during week 10-65, the feed efficiency in HFS fed mice, but not HFP fed mice, was significantly higher than in the REF group. In week 70-95, HFS and HFP fed mice lost body weight. For all groups, a reduction in feed efficiency was also evident during time.

The white adipose tissue depots, inguinal white adipose tissue (iWAT) and gonadal white adipose tissue (gWAT), reflected body weight, and mice fed the HFP diet were protected against an increase in adipose tissue mass compared to the REF fed mice (Fig 1C). As expected, the white adipose tissue increased with time in all groups.

Comparison of the survival curves between the different dietary groups until 50% survival, revealed a significant reduction in survival for mice fed the HFS diet compared to the REF group (Fig. 1D). In agreement with Keipert et al (22), a high protein:sucrose ratio attenuated high fat diet-induced weight gain, and furthermore, the survival of mice fed HFP was not statistically different from that of REF fed mice. All deceased animals were subjected to necropsy, but there was no systematic cause of death. Overall, the relationship between diets, adiposity and survival indicated that obesity, and not the high fat feeding, was linked to a reduction in survival as determined when 50% of the mice had died. Thus, a high fat diet only led to a significant reduction in survival when combined with a high sucrose intake that also increased body weight and fat mass. On the other hand, a high fat diet combined with a high protein intake prevented a significant reduction in survival, and in addition, also reduced body weight development compared to the HFS diet, and prevented a significant increase in fat mass compared to the REF fed mice.

A high protein:sucrose ratio diminishes high fat diet-induced accumulation of lipids in liver, but not a transient reduction in glucose tolerance

Obesity and high fat feeding are associated with hepatic steatosis. Mice fed a diet with high protein:sucrose ratio were protected against high fat diet-induced accumulation of triacylglycerol in the liver after 3 and 6 months of feeding (Fig. 2A). The HFP fed mice were also protected from a high fat diet-induced increase in liver weight compared to the REF fed mice, contrasting the increased liver weight and triacylglycerol accumulation observed for the HFS fed mice (Fig. 2A).

In agreement with previous studies in male mice (14, 30, 32), intake of a diet with high protein:sucrose ratio did not protect the mice against high fat diet-induced impairment of glucose tolerance at 3 months (Fig. 2B). Both groups of high fat fed mice displayed an impaired glucose clearance compared to mice receiving the REF diet, with the HFP fed mice exhibiting the largest area under the curve during the glucose tolerance test. However, at 18 months we observed no difference in glucose clearance between the groups (Fig. 2C).

Modulation of global gene expression in liver and adipose tissue during time is dependent on the protein:sucrose ratio

To achieve an overview of global changes in gene expression over time in relation to feed intake and composition, we performed digital gene expression (DGE) analyses on liver and two metabolically different adipose tissue depots, iWAT and gWAT. For iWAT and gWAT, PCA plots revealed a clear separation between the samples taken at different timepoints (6 vs. 18 months), while the separation according to the protein:sucrose ratio was less pronounced (Fig. 3A). For liver, only samples from HFP fed mice separated clearly after 18 months of feeding. In keeping with the clear clustering in the PCA of gene expression in liver from HFP fed mice at 18 months, 361 genes in the HFP fed mice, but only 15 genes in the HFS fed mice were differentially regulated between 6 and 18 months, demonstrating the impact of the HFP diet over time in liver. In the fat depots, the HFS diet caused a higher number of genes to be

differentially regulated between 6 and 18 months in iWAT (663+150) and gWAT (1014+199) compared to HFP feeding (iWAT (154+150) and gWAT (269+199)), indicating significant diet-dependent differences in expression of genes over time especially in adipose tissues (Fig. 3B).

A high dietary protein:sucrose ratio modulates long term effects on expression of genes involved in fatty acid metabolism, amino acid degradation and gluconeogenesis

To investigate if hepatic lipid accumulation was associated with changes in expression of genes involved in fatty acid synthesis and oxidation, we performed RT-qPCR analyses on RNA isolated from liver. In line with previous short-term studies (30, 32), hepatic expression of the lipogenic genes, *Acaca*, *Fasn* and *Scd1*, was reduced in the HFP fed mice after 3 months of feeding (Fig. 4A). However, after 6 months of HFP feeding only expression of *Scd1* was reduced, whereas expression of both *Scd1* and *Fasn* was reduced after 18 months of HFP feeding. This demonstrates that diet-induced changes in gene expression only persisted for some of the measured lipogenic genes during long term feeding, suggesting that a general lower capacity for *de novo* hepatic fatty acid synthesis in HFP fed mice was only maintained during the first 3 months of feeding. Still, the HFP fed animals remained less obese than the HFS fed animals throughout the study. By 3 months of feeding, the levels of mRNAs encoding enzymes involved in fatty acid oxidation (*Acox1*, *Cpt1a* and *Acadm*) and ketogenesis (*Hmgcs2*) were similar in low fat and high fat fed mice (Fig. 4B). By 6 months, expression of the genes involved in fatty acid oxidation, *Acox1* and *Cpt1a*, was up-regulated in the high fat fed mice compared to low fat fed mice, although this was not seen after 18 months. Thus, the protein:sucrose ratio strongly affected expression of genes involved in lipid metabolism during the first phases of feeding, but the difference between the HFS and HFP fed mice diminished over time.

A sustained increase in mRNA levels of genes involved in amino acid degradation (*Got1*, *Cps1*, *Agxt* and *Gpt*) (Fig. 4C) and gluconeogenesis (*Pck1*) (Fig. 4D) in liver was observed throughout the experiment in HFP fed mice indicative of an increased energy demanding amino acid catabolism in HFP fed animals compared to HFS fed animals. This is in keeping with the lower body weight and fat pad mass in the HFP fed animals.

High fat feeding and adipose tissue expansion are associated with low-grade inflammation in adipose tissue. HFS feeding led to increased expression of mRNA encoding inflammatory markers, *Ccl2* (chemokine (C-C motif) ligand 2) and *Serpine1* (serine (or cysteine) peptidase inhibitor, clade E, member 1) (Fig. 4E), but not in macrophage infiltration markers, *Cd68* and *Emr1* (EGF-like module containing mucin-like, hormone receptor-like sequence 1) at 3 and 6 months (Fig. 4F). This increase was attenuated in mice fed the diet with high protein:sucrose ratio. However, after 18 months of feeding, expression levels were similar in all groups, except that expression of *Cd68* and *Emr1* mRNA was elevated in HFP fed mice compared with HFS and REF fed mice.

Host age and dietary fat are major drivers of gut microbiota composition

Whereas changes in gene expression can reflect modulation of metabolic processes, several recent studies have suggested that the gut microbiota plays an important role in diet-induced obesity (1, 8, 24, 40, 47, 48) and associated metabolic disorders (24, 37). To investigate how host age, dietary fat, protein:sucrose ratio and obesity impacted on the bacterial composition in the gut, bacterial DNA from feces (n=3-6 per diet group and time point) was analyzed using the Mouse Intestinal Tract Chip (MITChip) (13, 45).

Firstly, as expected the high fat diets (HFDs) resulted in a distinct microbiome clustering compared to the REF diet (Fig. 5). Comparison of the microbiomes in the three dietary groups using redundancy analysis (RDA) (Fig. 5) confirmed that particularly the high fat content of

the HFS and HFP diets affected the gut microbiome composition. Separation of the HF-diets from the REF-diet was predominantly driven by phylotypes within the *Lactobacillus* genus (*L. delbrueckii*, *L. plantarum*) which were common for the HF diets at all three time-points (Fig. 5). The protein:sucrose ratio also affected the gut microbiome composition (Fig. 6), where a high abundance of *E. cylindroides* and the genus-like group *Eggerthella et rel* characterized mice fed the HFS diet, while phylotypes within the *Clostridiaceae* family (*Anaerovorax*, *Bryantella*, *C. herbivorans*, *C. sphenoides*, *C. leptum* and *C. symbiosum*) characterized mice fed the HFP.

Secondly, the aging of the mice also resulted in distinct differences in the microbial communities. The *Firmicutes/Bacteroidetes* (F/B) ratio decreased with age in the mice fed REF and HFP diets, while no significant change was observed in mice fed the HFS diet (not shown). RDA plots showed distinct separation of the microbiomes from 3 over 6 to 16 months of feeding, for all three diets (Fig. 7A, B and C). Interestingly, while there was no common bacterial phylotypes driving the age-related separation of the gut microbiomes across the diets at three months, several phylotypes (*Sphingomonas*, *Desulfovibrio*, *Olsenella*, *Akkermansia muciniphila*) were driving separation in relation to HFDs at six months, and two bacterial phylotypes (*Porphyromonodaceae* and *Clostridiaceae*) were driving the separation of the gut microbiome composition for all three diets at 16 months. Taken together this suggested that host age was a major driver of microbiome composition independent of diet. This was further supported by a weaker separation of the gut microbiomes between HFS and HFP with host age as shown in figure 7.

Overall, the most pronounced changes in the composition of the gut microbiota were observed in relation to age (Fig. 7) and dietary fat content (Fig. 5). Although gut microbiomes separated in response to changes in the dietary protein:sucrose ratio (Fig. 6), this was not as prominent as the impact of a HFD.

339

340 **Discussion**

341 Experiments using rodent models of high fat diet-induced obesity have provided considerable
342 insight into obesity-related metabolic disorders (20). However, by using such models it is
343 difficult to distinguish obesity related effects from effects elicited by high fat feeding. To
344 discriminate between these factors, we took advantage of the observation that obesity is
345 prevented if the increase in dietary fat is accompanied with an increased protein:sucrose ratio
346 (12, 14, 30, 32, 33, 36). Short term effects of high protein diets are well described (12, 14, 30,
347 32, 33, 36), but less is known in relation to long term effects. Therefore, we fed mice high fat
348 diets with both a high and low protein:sucrose ratio throughout life to investigate the long-
349 term effects on metabolism and survival.

350 As previously demonstrated in short term studies with male mice (14, 30, 32), high protein
351 feeding protected mice from developing high fat diet-induced obesity. This study
352 demonstrates that high protein feeding has a persistent long term effect preventing high fat
353 diet-induced obesity. The lean phenotype of the HFP fed mice, together with the obese HFS
354 fed mice, provides a unique opportunity to distinguish the impact of high fat feeding from that
355 of obesity development in relation to survival and gut microbiota composition.

356 The mice fed a HFS diet exhibited a significant reduction in survival compared to the REF fed
357 mice. Moreover, the mice fed a high protein diet were protected from a significant reduction
358 in survival compared to the REF fed mice, in agreement with a previous study (22),
359 demonstrating that an increase in the protein:sucrose ratio protects mice against high fat diet-
360 induced reduction in survival. In line with our observation that an initially increased caloric
361 intake and expansion of adipose tissue were associated with a reduced survival in HFS fed
362 mice, Keipert et al (22) concluded that the harmful effects of high fat diets on longevity were

linked to early and rapid obesity development. Thus, the results from their study and our study indicate that the state of obesity, rather than high fat feeding is associated with reduced survival. In contrast to these studies, the use of a matrix-based approach for changing the macronutrient composition of the feed concluded that lifespan increased with a decrease in the protein:carbohydrate ratio (43). It is possible that the discrepancies between our study and that of Solon-Biet et al. in relation to the effect of protein:carbohydrate ratio on lifespan may be due to the fact that we used a higher protein:carbohydrate ratio, mainly due to a lower carbohydrate content in our HFP diet. Never the less, in agreement with our study, a low protein:carbohydrate ratio was also associated with increased body fat and hepatic lipid accumulation.

The timing of a high protein intake in humans has been considered in relation to lifespan, where a high intake of animal proteins during middle age (50-65 year) was reported to be associated with an increase in all-cause mortality linked to a higher level of IGF-1, while a decreased mortality was reported in older persons (above 66 years) (25). However, a high protein intake in this study was defined as 20 energy per cent or more and a low protein intake defined as less than 10 energy per cent, while all the diets from our mouse experiment had a protein content close to or above the definition for high protein intake in the human study. The impact of IGF-1, in addition to insulin signaling, has also in earlier studies been related to lifespan (2), where an extended lifespan and reduced fat mass have been demonstrated in mice with disruption of the insulin receptor in adipose tissue (3). In earlier short term studies, the insulin level in HFP fed mice was demonstrated to be significantly reduced compared to a HFS diet (30, 32). Thus, a reduction in insulin levels by a HFP diet and extended lifespan with reduced insulin signaling and fat mass, are thereby in line with the protection from a high fat diet-induced increase in mortality of the HFP fed mice in our study.

An impact of gender on lifespan has been demonstrated in several studies ((29, 39), where an extended lifespan is generally reported in females compared to males. As IGF-1 has been linked to survival, it is of interest that there are reported gender differences in lifespan of mice with heterozygous knockout of the insulin-like growth factor type 1 receptor (21). In the study of Solon-Biet et al. (43) using both male and female mice, no differences in lifespan in relation to diet were observed, whereas gender-specific differences in reproductive function were observed (44).

Female mice are largely protected against metabolic disorders, despite obesity and increased serum TAG and cholesterol when fed a high fat diet (35). However, similar to our earlier findings in male mice, high fat feeding in combination with high amounts of sucrose leads to impaired glucose tolerance (14, 30). A high protein:sucrose ratio did not protect the mice against high fat diet-induced impaired glucose tolerance when mice were fed for 3 months, despite protection against obesity. Supporting this, expression of *Pepck*, encoding the rate-limiting enzyme in hepatic gluconeogenesis was increased in HFP fed mice. In agreement with a similar report of a transiently impaired glucose homeostasis in high fat high protein fed mice (22), we observed no difference in glucose clearance between the groups after 18 months.

A high protein:sucrose ratio protected the mice against high fat diet-induced accumulation of triacylglycerol in the liver for at least 6 months. Moreover, the high protein:sucrose ratio alleviated the initial high fat diet-induced expression of inflammatory markers in adipose tissue. In line with an earlier report that female mice were protected against high fat diet-induced macrophage infiltration (35), we did not detect increased expression of macrophage selective markers in samples obtained after 3 and 6 months of feeding. However, after 18 months of feeding, expression of the macrophage infiltration markers, *Cd68* and *Emr1*, in adipose tissue was surprisingly higher in HFP fed mice than in HFS and REF fed mice.

Digital gene expression analysis in adipose tissue and liver demonstrated different patterns of expression over time in response to the dietary protein:sucrose ratio. Further RT-qPCR analyses of liver mRNA indicated that a high protein:sucrose ratio led to increased amino acid catabolism and ureagenesis, as well as reduced lipid synthesis during the first months of feeding, in agreement with our earlier short term experiments in male mice (14, 30, 32). Our study also demonstrated that the increased expression of genes linked to amino acid degradation and gluconeogenesis was maintained in the HFP fed mice after 18 months of feeding. By contrast, the changes in expression of genes involved in fatty acid synthesis were not sustained throughout the study.

As the gut microbiome is modulated in response to diet (6, 26), obesity (1, 8, 40, 47, 48), inflammation (5) and host age (46, 52), it was of interest to follow how the different diets affected the gut microbiota over time. In agreement with previous reports (6, 51), we observed that dietary fat was a strong driver of the composition of the gut microbiota. Moreover, we found that the protein:sucrose ratio significantly affected microbiome composition. The dietary protein:sucrose ratio regulates the development of obesity in mice (30), and an obese phenotype may in itself impact the microbiota (26). However, we observed an increasing difference in obesity over time between HFS and HFP fed mice, which occurred in parallel with a decrease in differences between the gut microbiomes of these groups, suggesting that obesity-independent changes in the gut microbiome occurred in response to ageing and dietary protein:sucrose ratio. Thus, a distinct microbiome composition was observed at 3, 6 and 16 months of age within all three dietary groups, confirming that age-dependent changes occurred in the gut microbiome. Interestingly, while the bacterial phylotypes driving the longitudinal differences within each dietary group were not the same for the three diets at 3 months, the inter-dietary differences diminished with time, suggesting that host age is a major driver of the gut microbiome composition independently of diet.

437

438 Age-related changes reported in elderly people (7) may be associated with decreased capacity
439 for energy harvest from the food (48). In our study, *Akkermansia muciniphila*, a bacterium
440 previously associated with leanness and inversely correlated with body weight (9), was
441 driving the difference in gut microbiome composition at 6 months for animals fed the two HF-
442 diets. Interestingly, at this time-point the HFS fed mice, but not the HFP fed mice, were
443 significantly more obese than the REF fed mice, suggesting that *Akkermansia* was affected by
444 dietary fat rather than by bodyweight.

445

446 Overall, high dietary fat content and host age, independent of obesity, appeared to be the two
447 most important drivers of the gut microbiota composition in this study. In contrast to this, the
448 survival of mice was significantly reduced in the obese HFS fed mice compared to the low fat
449 REF group, but not in the lean HFP fed mice, suggesting obesity to be more important for
450 survival than the high dietary fat intake. This is also in line with the changes related to
451 progression of colon cancer (28), reported to be induced by high fat feeding, but resulting
452 from obesity and not diet. These results highlight the importance of separating the impact of
453 high fat feeding from that of obesity. In the present study the dietary fat content was a more
454 important driver of the gut microbiota composition than obesity, as the mice fed a HFS and
455 HFP diet had similar gut microbiota profiles despite differences in obesity development.

456

457 In conclusion, our findings support the notion that reduced survival in response to high fat
458 feeding is linked to obesity development in mice fed a diet with a low protein:sucrose ratio. A
459 high protein:sucrose ratio in the diet protected against high fat diet-induced obesity, hepatic
460 lipid accumulation and a significant reduction in survival. We observed the greatest diet-
461 dependent differences in gene expression during the early period of growth. Over time these

differences diminished, pointing to the importance of the early growth period. We detected marked effects on the composition of the gut microbiota over time, and fat content in the diet and age rather than adiposity or protein:sucrose ratio were observed to be major drivers shaping the gut microbiota.

Acknowledgments

This work was supported by the Danish Natural Science Research Council, the Novo Nordisk Foundation, the Carlsberg Foundation, and National Institute of Nutrition and Seafood Research (NIFES). Parts of the work were supported by the Gut, Grain and Greens (3G) center, funded by the Danish Council for Strategic Research (Grant no 11-116163).

Disclosures

The authors declare no financial or non-financial competing interests.

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Figure captions

Figure 1. Body weight development and survival. Body weight gain (A), feed efficiency (Mcal energy eaten per g body weight gain) (B), white adipose tissue depots; inguinal white adipose tissue (iWAT) and gonadal white adipose tissue (gWAT) dissected out after 3, 6 and 18 months of feeding (C) and Kaplan-Meier survival curves (D) of mice fed a low fat reference diet (REF), high fat diet with sucrose (HFS) or protein (HFP). Significant overall effects of diet on body weight ($p < 0.001$ for all comparison) and time ($p = 0.0001$ for all diets), in addition to weekly differences between diets ($p < 0.05$) were detected on body weight development. Lower-case, upper-case and italic letters denote significant differences within the 3, 6 and 18 months samples, respectively. *Denotes significant difference in survival between REF and HFS ($p = 0.0148$).

Figure 2. The effect of high fat feeding on hepatic lipid accumulation and glucose tolerance. Triacylglycerol (TAG) concentration (mg/g) in liver and liver weight of mice fed a low fat reference diet (REF), high fat diet with sucrose (HFS) or protein (HFP) (A). Lower-case, upper-case and italic letters denote significant differences within the 3, 6 and 18 months samples, respectively. Fasted blood glucose (16h), oral glucose tolerance test (OGTT), and area under the curve (AUC) for OGTT after 3 months (B) and 18 months (C) of feeding with REF, HFS and HFP diets. The OGTT and AUC panels were analyzed using a repeated measures two-way ANOVA and one-way ANOVA, respectively, followed by Bonferroni corrected pair-wise comparisons, $n = 8$. Different letters denote significant differences.

Figure 3. Digital gene expression profiling on gonadal adipose tissue, inguinal adipose tissue and liver. PCA plots showing distinct clustering of samples mostly dependent on time (A) and with corresponding Venn diagrams showing the degree of overlapping gene expression between the diets ($FDR \leq 5\%$) (B).

Figure 4. Gene expression in liver and inguinal white adipose tissue after 3, 6 and 18 months of REF, HFS and HFP feeding. Expression of genes involved in fatty acid synthesis; *Acaca*, *Fasn*, *Scd1* and *Srebf1* (A), fatty acid oxidation; *Acox1*, *Cpt1a*, *Acadm*, and *Hmgcs2* (B), amino acid degradation; *Got1* and *Cps1* (C), and gluconeogenesis; *Ppargc1a* and *Pck1* (D) was measured in liver. Expression of genes involved in inflammation; *Ccl2* and *Serpine1* (E) and macrophage infiltration; *Cd68* and *Emr1* (F) was measured in inguinal white adipose tissue (iWAT). Lowercase, uppercase and italic letters denote significant difference between diets within the 3, 6 and 18 month time-point, respectively (n=4-8).

Figure 5. The effects of diet on the composition of the gut microbiota. Redundancy analysis, RDA, of microbiota composition in fecal samples collected from the cages at 3 months (A), 6 months (B) and 16 months (C) of feeding. Animal weight (red arrow) and the different diets (REF (black), HFS (red), HFP (blue)) were included as explanatory variables. These variables explain 42.6% (A), 45.9% (B) and 44.4% (C) of total variation. The relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best fitting 15 genus groups are shown in the plots.

Figure 6. Comparison of the gut microbiota between mice fed the HFP and HFS diets. Redundancy analysis, RDA, of microbiota composition in fecal samples collected from the cages at 3 months (A), 6 months (B) and 16 months (C) of feeding the high fat diets (high fat and high sucrose (HFS) and high fat and high protein (HFP)). The explanatory variables are the weight of the animals (red arrow), and the HFP (blue) and HFS (red) diets. These variables explain 19.7% (A), 25.5% (B) and 26.6% (C) of total variation. The relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best fitting genus groups are shown in the plots.

Figure 7. The effect of age on the composition of the gut microbiota. Redundancy analysis, RDA, of microbiota composition in fecal samples collected from the cages (n=3-7 cages per diet) at 3, 6 and 16 months of feeding REF (A), HFS (B) or HFP (C) diet. The explanatory variables are the weight of the animals and the time points. These variables explain 39.1% (A), 45.3% (B) and 47.9% (C) of total variation. The relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best fitting 15 genus-like groups are shown in the plots.

712 Table 1: Diet composition.

	High fat diets		
	Low fat (REF)	High sucrose (HFS)	High protein (HFP)
Protein (g/kg)	200	200	540
Casein	200	200	540
L-Cystine	3	3	3
Carbohydrate (g/kg)	619.5	439.5	99.5
Corn starch	529.5	9,5	9,5
Sucrose	90	430	90
Fat (g/kg)	70	250	250
Soybean oil	70	70	70
Corn oil		180	180
Energy (kcal/kg)	3908	4808	4808
Protein (E%)	20.5	16.6	44.9
Carbohydrate (E%)	63.4	36.6	8.3
Fat (E%)	16.1	46.8	46.8
P:C ratio	0.32	0.46	5.43

713 Equal for all diets: Cellulose (50 g/kg), Choline bitartrate (2.5 g/kg), Mineral mix (45 g/kg),

714 Vitamin mix (10 g/kg), tert-Butylhydroquinone (0.014 g/kg).

715 Table 2. Primers (from Rajilic-Stojanovic et al., 2009)

<i>Primer name</i>	<i>Sequence</i>	<i>Application</i>
<i>T7prom- Bact-27-F</i>	5'-TGA ATT GTA ATA CGA CTC ACT ATA GGG GTT TGA TCC TGG CTC AG-3'	MITChip
<i>Uni-1492-R</i>	5'-CGG CTA CCT TGT TAC GAC-3'	MITChip

716

Figure 1

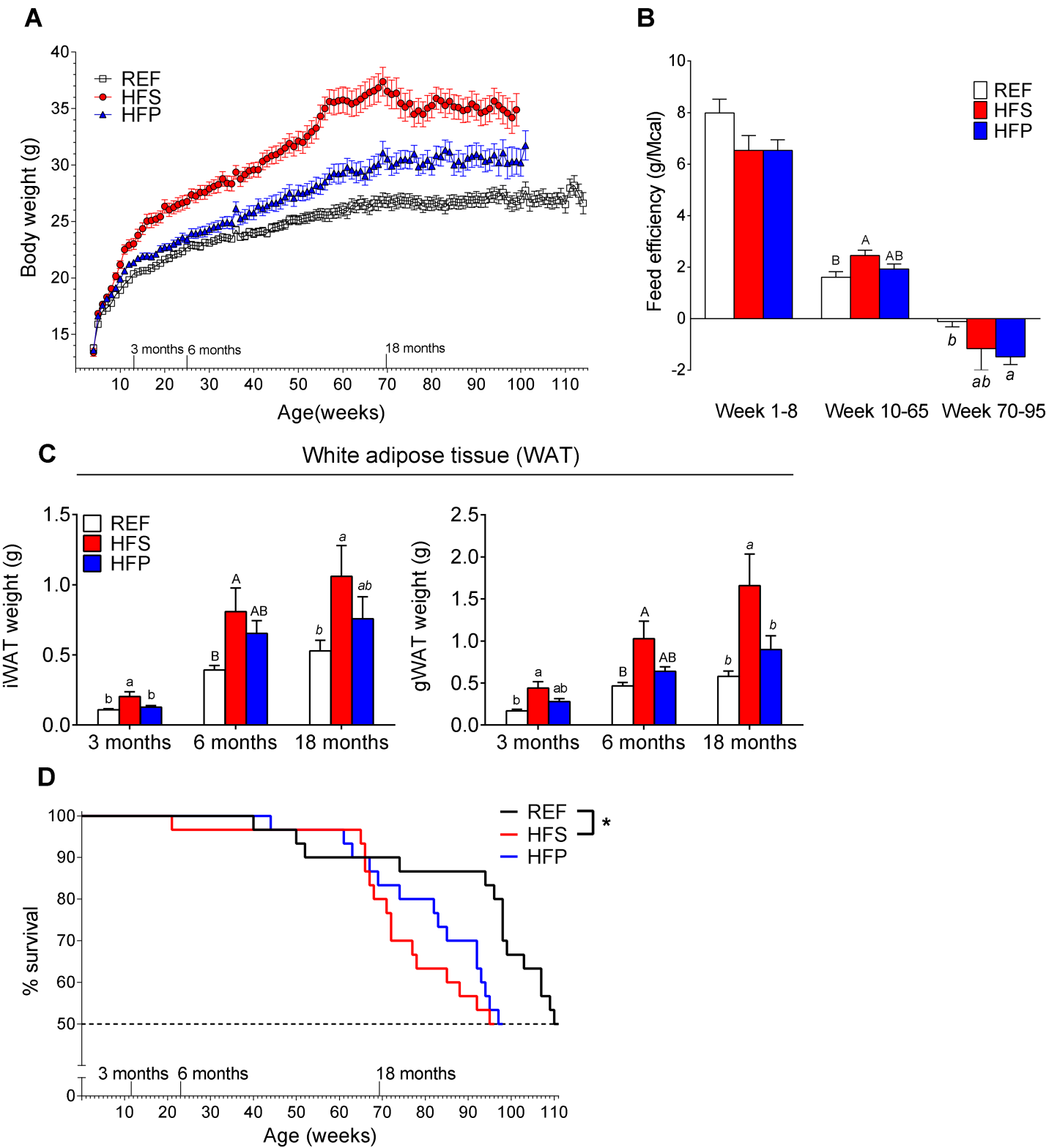


Figure 2

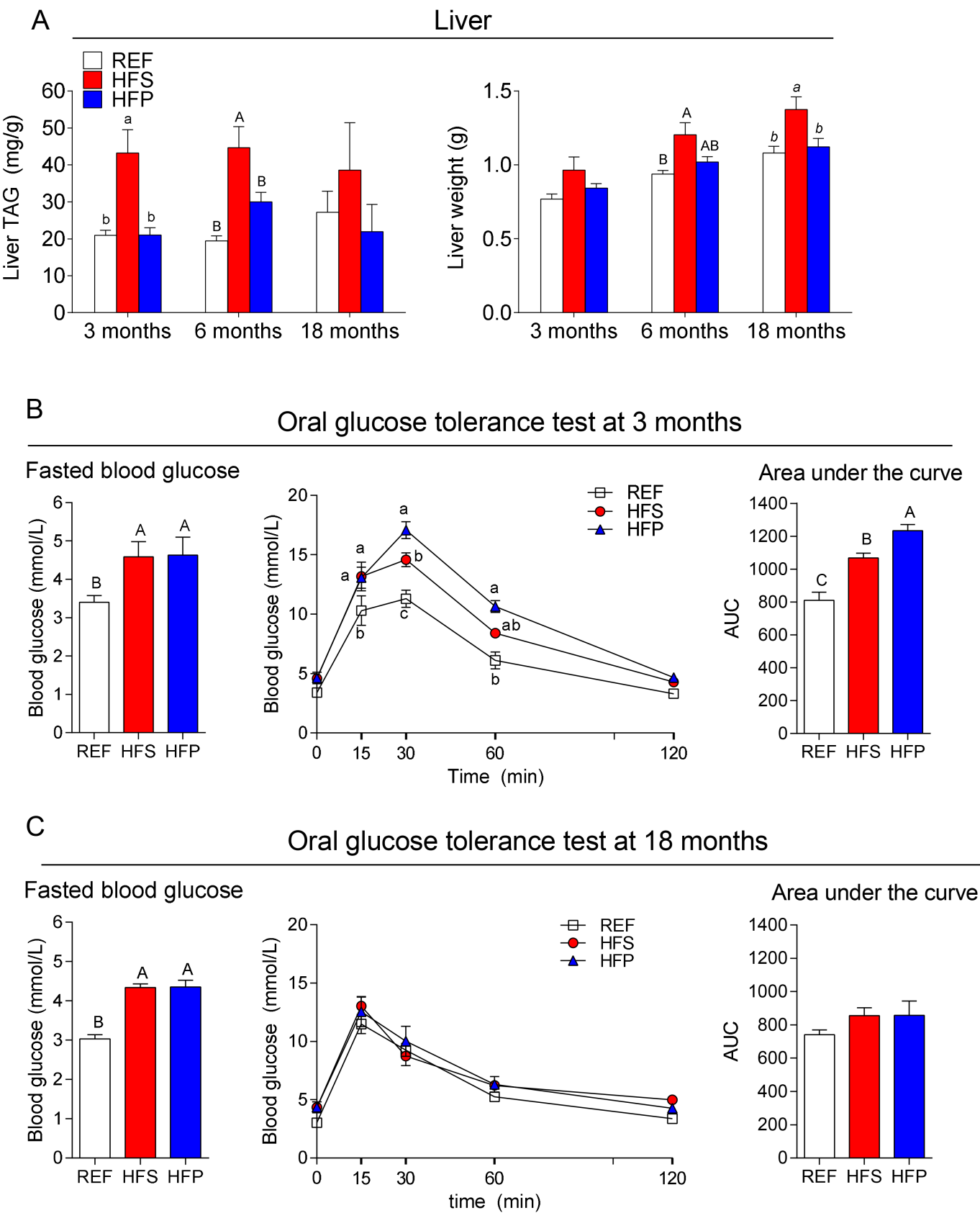
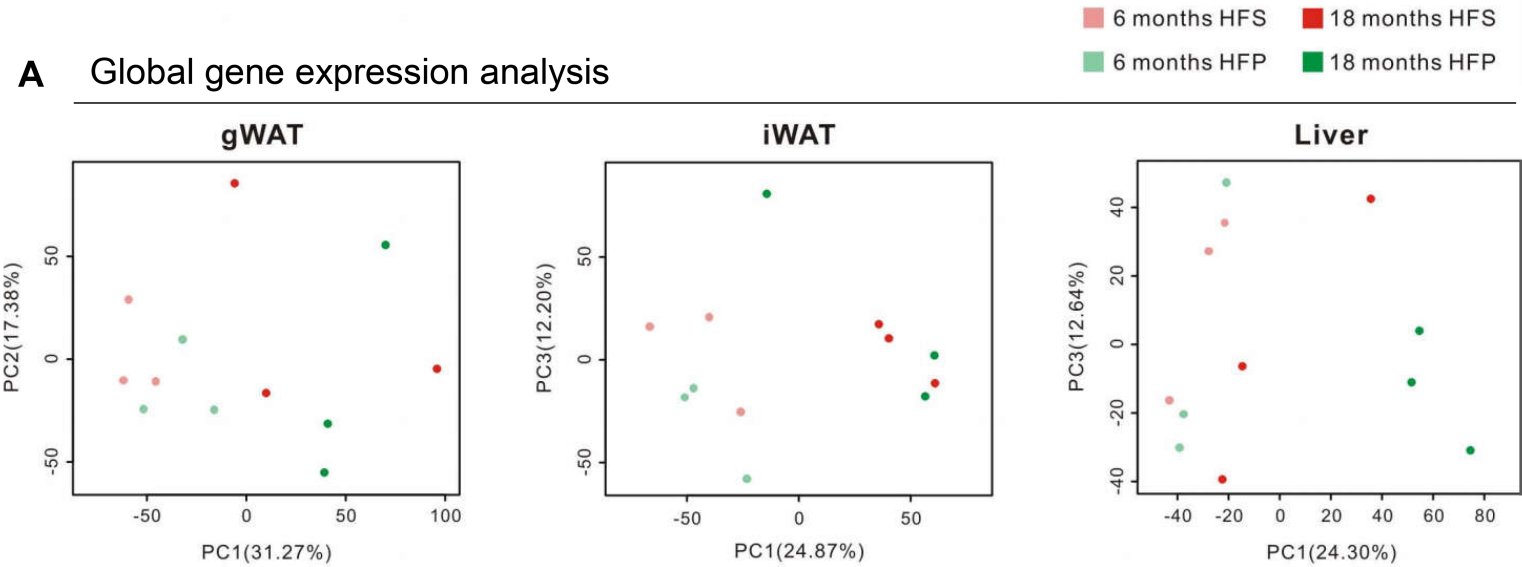


Figure 3

A Global gene expression analysis



B Number of differentially regulated genes

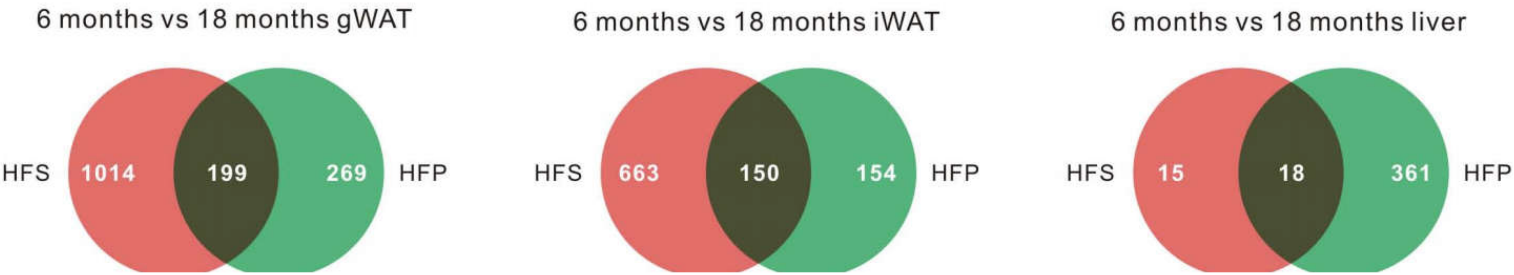


Figure 4

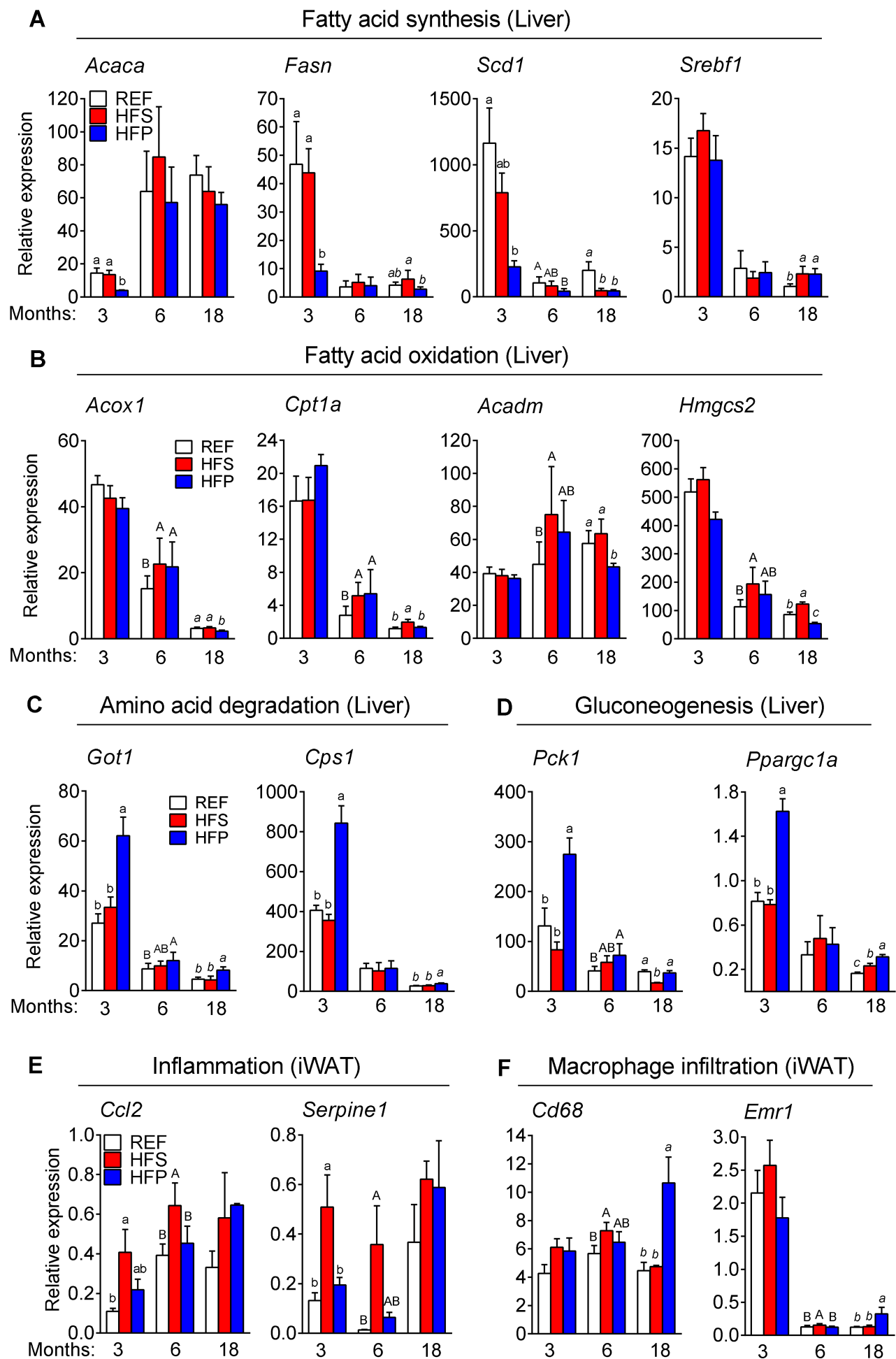
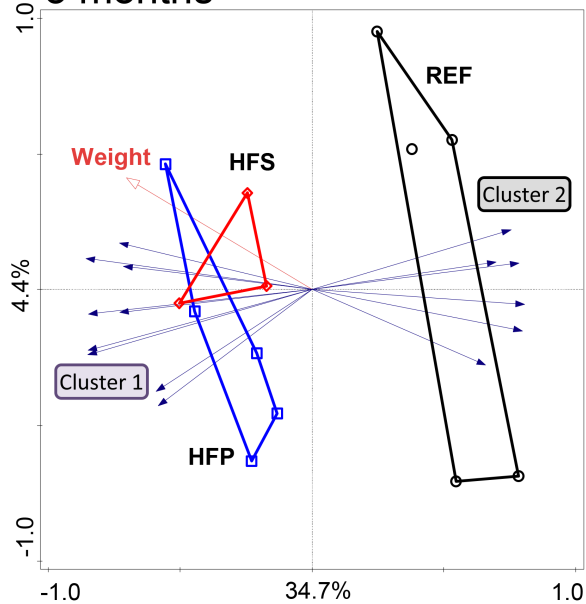


Figure 5

A 3 months



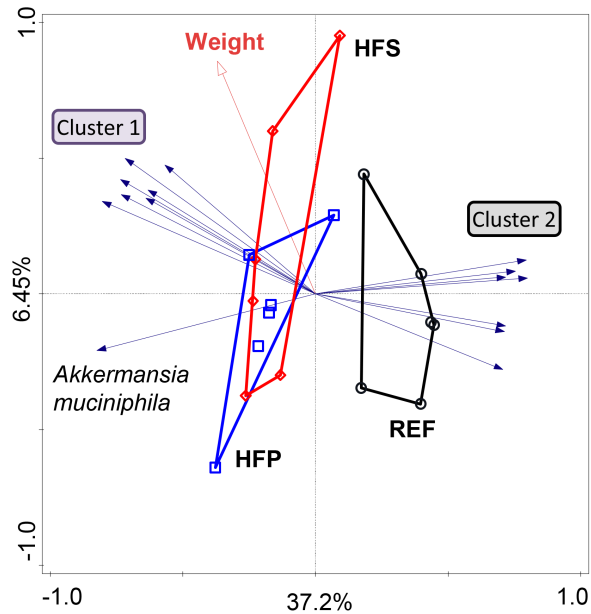
Cluster 1

Labrys methylaminiphilus et rel.
Fibrobacter succinogenes et rel.
Vibrio et rel.
Lactobacillus acidophilus et rel.
Lactobacillus plantarum et rel.
Lactobacillus gasseri et rel.
Lactobacillus delbrueckii et rel.
Anaerovorax et rel.
Clostridium leptum et rel.

Cluster 2

Staphylococcus aureus et rel.
Olsenella et rel.
Eggerthella et rel.
Bilophila et rel.
Desulfovibrio et rel.
Akkermansia muciniphila

B 6 months



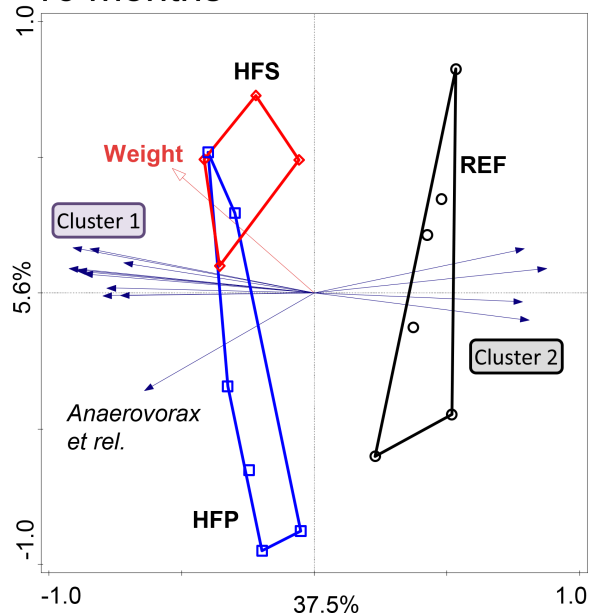
Cluster 1

Anaerovorax et rel.
Lactobacillus delbrueckii et rel.
Lactobacillus plantarum et rel.
Clostridium sphenoides et rel.
Bryantella et rel.
Lactobacillus acidophilus et rel.
Clostridium leptum et rel.

Cluster 2

Coprobacillus et rel.
 - *Clostridium ramosum* et rel.
Solobacterium moorei et rel.
Porphyromonas asaccharolytica et rel.
 Unclassified Clostridiales XVI
Eubacterium rectale et rel.
 Unclassified Mollicutes
Eubacterium cylindroides et rel.

C 16 months



Cluster 1

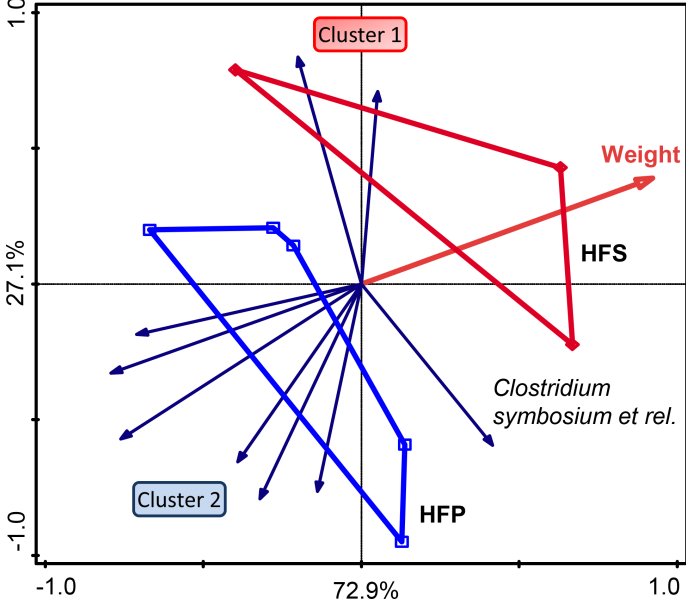
Clostridium leptum et rel.
Olsenella et rel.
Lactobacillus delbrueckii et rel.
Lactobacillus gasseri et rel.
Vibrio et rel.
Lactobacillus plantarum et rel.
Fibrobacter succinogenes et rel.
Labrys methylaminiphilus et rel.
Bryantella et rel.
Pasteurella

Cluster 2

Unclassified Clostridiales XIVa
 - close to *Anaerostipes caccae*
Ruminococcus obeum et rel.
Rikenella et rel.
Turicibacter et rel.

Figure 6

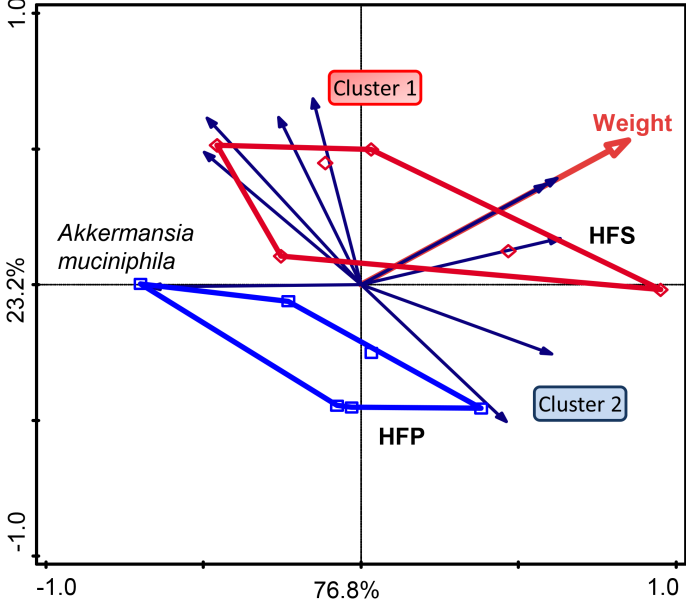
A 3 months



Cluster 1
Eubacterium cylindroides et rel.
Eggerthella et rel.

Cluster 2
Sutterella wadsorthia et rel.
Sphingomonas et rel.
Anaerovorax et rel.
Lachnobacillus bovis et rel.
Clostridium herbivorans et rel.
Uncultured Clostridiales

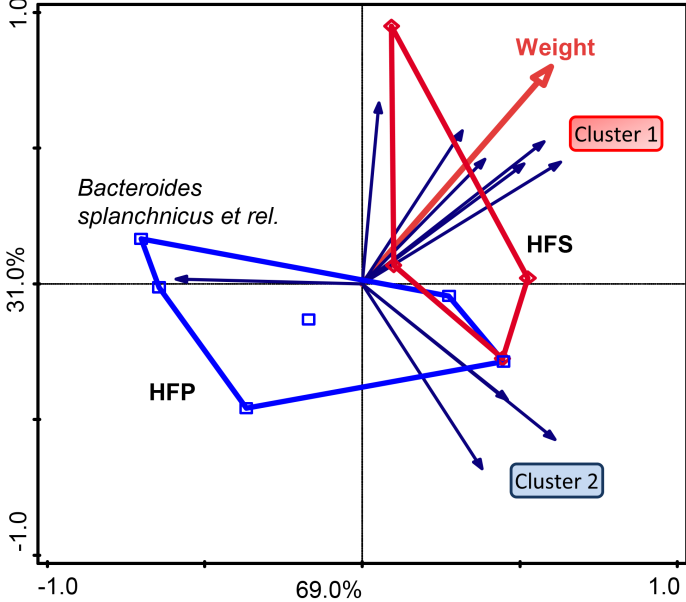
B 6 months



Cluster 1
Helicobacter
Anaerotruncus et rel.
Eubacterium cylindroides et rel.
Turicibacter et rel.
Propionibacterium
Pasteurella
Lactobacillus delbrueckii et rel.

Cluster 2
Clostridium sphenoides et rel.
Clostridium leptum et rel.

C 16 months



Cluster 1
Eggerthella et rel.
Eubacterium cylindroides et rel.
Collinsella
Solobacterium moorei et rel.
Acholeplasma et rel.
Catenibacterium

Cluster 2
Unclassified Clostridiales
-Close to Clostridium symbiosum et rel.
Olsenella et rel.
Bryantella et rel.

Figure 7

